INTRODUCTION

1. Metal ions in biological systems

Coordination compounds are widely present in the mineral, plant and animal world and are known to play important functions in diverse area such as analytical chemistry, metallurgy, biological systems, industry and medicine. Naturally occurring coordination compounds are vital to living organisms. Perhaps, the most significant metal complex in our body is the hemoglobin. Erythrocytes are red due to the presence of hemoglobin, the conglomerate macromolecule responsible for oxygen transport. Hemoglobin contains iron-porphyrin complexes, its role as an oxygen carrier being related to the ability of the iron atoms to coordinate oxygen molecules reversibly \[1\].

Hemoglobin is composed of four subunits, each consisting of a non-protein group heme surrounded by a coiled protein (globin). Each heme group contains of an iron (Fe\(^{II}\)) ion surrounded by a heterocyclic ring. The iron center has six potential coordination sites, four of which are occupied by porphyrin nitrogens (N). The fifth coordination site (below the plane of the ring) covalently bonds with a histidine (His) residue from the F8 position of its respective globin chain. The sixth coordination site (above the plane of the ring) is where all the "action" occurs. This is the place oxygen and other small molecules transiently bind to the iron atom \(\text{(Figure 1)}\). Other biologically important coordination compounds include chlorophyll, a magnesium-porphyrin complex and vitamin B\(_{12}\) or cyanocobalamine, a complex of cobalt(III) with a macrocyclic ligand known as corrin \(\text{(Figure 2)}\)[2].
Figure 1. Structure of haemoglobin (a) A computer graphic of structure of hemoglobin, (b) heme, (c) coordinating site in hemoglobin
Figure 2. Chemical structure of chlorophyll (a) and cyanocobalamine (b)
With the application of new and sophisticated machines to study biological and biochemical systems, it is increasingly recognized that metal ions are involved in various cellular and sub-cellular functions. Today, it is known that metals are important ingredients in living cells, just as the organic molecules. For instance, the divalent magnesium and calcium ions play important regulatory roles in cells. The divalent cations Zn\(^{2+}\), Ca\(^{2+}\) and Mg\(^{2+}\) prevent cytotoxicity and *in vivo* antagonize Cd-induced carcinogenesis. The transport of iron and other metal ions by the blood plasma is achieved through the formation of protein complexes\([3]\). Lack of body iron is common in cancer patients and it is associated with complications in surgery and in animal experiments. Copper is recognized as an essential metalloelement and is primarily associated with copper-dependent cellular enzymes\([4]\).

In the fascinating field of ‘metals in biology’, by virtue of direct interactions with amino acid side-chains within polypeptide chains, metals play unique and critical roles in biology, promoting structures and chemistries that would not otherwise be available to proteins alone\([5]\). Metal ions play essential roles in about one third of enzymes. Many enzymes that regulate biological processes are metal complexes (metalloenzymes); for example, carboxy peptidase, a hydrolytic enzyme important in digestion, contains a zinc ion coordinated to several amino acid residues of the protein. Another enzyme, catalase, which is an efficient catalyst for the decomposition of hydrogen peroxide, contains iron-porphyrin complexes. In both cases, the coordinated metal ions are probably the sites of catalytic activity. These ions can modify electron flow in a substrate or enzyme, thus effectively controlling an enzyme-catalyzed reaction. They can serve to bind and orient substrate with respect to functional groups in the active site, and they can provide a site for redox activity if the metal has several valence states\([6]\).
Metal ions are generally positively charged and act as electrophiles, seeking the possibility of accepting electron pairs with other atoms so that a bond or dipole-dipole interaction can be formed. They behave rather like hydrogen ions. However, metal ions often have positive charges greater than d, and have a larger ionic volume so that they can accommodate many ligands around them at the same time. In addition, metal ion concentrations can be high at neutral pH values, while hydrogen ion concentrations are, by the definition of pH, low at these values [7].

Ligands are the atoms or group of atoms that are bonded to the metal ion, generally in an electrostatic manner. They are usually neutral or negatively charged and they donate electron density to the metal ion. The coordination number of a metal ion, that is, the number of ligand atoms bound to it, is viewed in terms of concentric spheres; the inner sphere containing those atoms in contact with the metal ion, the second sphere containing those in contact with the inner sphere ligand atoms. The number of atoms in these spheres will depend on the size of the metal ion and the sizes of the ligand atoms [8].

The charge distribution in the active site of an enzyme is designed to stabilize the transition state of the catalyzed reaction relative to that of the substrate. In enzyme-catalyzed reactions it is essential that the reactants be brought together with the correct spatial orientation, otherwise the chance of the reaction taking place is diminished and the reaction rate will be too low. The electrostatic environment in the active site is a major factor that serves to guide the substrate to the binding site in the correct orientation. Metal ions can assist in this process, often binding groups in a stereochemically rigid manner, thereby helping to control the action of the enzyme. Thus, an enzyme will bind its substrate in such a manner that immobilization and alignment, ready formation of the transition state of the reaction to be catalyzed, and then easy
release of the product will result; metal ions often help in accomplishing this process [9].

The enzyme provides an arrangement of side-chain functional groups having an appropriate sized hole with the preferred groups on enzyme side chains needed to bind the required metal ion. The optimal number of such binding groups is chosen for the particular metal ion, together with the appropriate hydrophobic or hydrophilic environment in the binding site [10]. Metal ions may be bound by main-chain amino and carbonyl groups, but specific binding is achieved by the amino acid side chains. No set of general rules exists that describes how a given metal ion will behave in an enzyme. Now that many crystal structures of proteins are being studied by X-ray diffraction, information on the binding of metal ions in the active sites of enzymes is available and should provide clues to the mechanism of action of the enzyme [11].

It is less well known than the fact that metal ions are required in biology is their role as pharmaceuticals. Two major drugs based on metals that have no known natural biological function, cisplatin (Pt) and auranofin (Au), are widely used for the treatment of genitourinary and head and neck tumours and of rheumatoid arthritis, respectively (Figure 3). In addition, compounds of radioactive metal ions such as $^{99}$Tc and complexes of paramagnetic metals such as Gd(III) are now in widespread use as imaging agents for the diagnosis of diseases [12].
Figure 3. Chemical structure of cisplatin (1), auranofin (2) and carboplatin (3)

Cisplatin (1), is perhaps the best known example of a small molecule metal-containing drug. Its use and effectiveness in cancer chemotherapy since the entry into the clinic in the late 1970s has been thoroughly documented. Cisplatin is cited for treatment of germ-cell cancers, gestational trophoblastic tumours, epithelial ovarian cancer, and small cell lung cancer as well as for palliation of bladder, cervical, nasopharyngeal, esophageal, and head and neck cancers. Despite this success, there is still a limited range of tumours sensitive to cisplatin intervention—some cancers are inherently resistant. The side effects of cisplatin treatment are severe and include the dose-limiting nephrotoxicity, neurotoxicity, ototoxicity, and emetogenesis\cite{13}.

The “second-generation” compounds based on the cisplatin structure were developed in attempts to reduce toxicity and/or expand the range of
useful anticancer activity. Carboplatin ((3), Figure 3) entered the clinic in 1998, principally in response to the necessity to reduce the toxic side effects of the parent drug. Despite this lower toxicity, carboplatin is essentially active in the same set of tumours as cisplatin and a broader spectrum of activity is not indicated. For some tumours, cisplatin appears to be therapeutically more effective than carboplatin (germ cell tumours, head and neck, and bladder) whereas for lung cancer and ovarian cancer effectiveness is comparable [14].

Since the advent of cisplatin in the clinic, the consistent goals for drug development have been improvement of toxicity profile, circumvention of resistance, and expansion of the tumors sensitive to treatment by cisplatin. To achieve these goals, modified versions of cisplatin and a large number of non-platinum complexes are explored for their cytotoxicity. In the past few years, for easy accessibility and less toxicity, complexes based on earlier transition metals, particularly Cu, have also attracted considerable interest [15].

Copper complexes have been shown to possess a broader spectrum of activity and a lower toxicity than platinum drugs and are suggested to be able to overcome inherited and/or acquired resistance to cisplatin [16]. These features are consistent with the hypothesis that copper complexes possess mechanism(s) of action different from platinum drugs that covalently bind to DNA. However, little information is available on the molecular basis for the mode of action of copper complexes. At present, most investigations still focus on the ability of these complexes or fragments thereof, to interact with DNA. However, other cellular constituents such as to poisermerases or the proteasome multiprotein complex are emerging as new putative targets [17]. Since 1969, copper has been found to possess high DNA binding affinity. Analogously to what has been widely illustrated for cisplatin, [18]. This binding was dependent on copper complex size, electron affinity, and geometry of the formed adduct, inducing an irreversible modification of the DNA conformational structure. According to these observations, a high number of
copper complexes has been and is still being tested as DNA-targeting agents [19].

Metal complexes have tremendous potential use as anticancer agents, due to their wide and diverse structural types, varied ligand bonding modes and a large number of metal complexes have been tested as anti-cancer drugs in an effort to find compounds with higher selectivity and lesser side effects than those commercially available. Due to unique magneto-structural correlations resulting from the interaction with macromolecules present in living cells, metal complexes exert a range of biological activities having therapeutic values and many of them successfully cleared clinical trials. The term metallodrug is used to describe such compounds and a number of them are already in use to treat many diseases including different types of cancer[20].

2. Curcuminoids and curcumin

The term curcuminoids represent a group of structurally related compounds present in the roots and shoots of herbaceous plant Curcuma Longa L (turmeric) and several other related Curcuma species of the family Zingiberaceae. Turmeric has traditionally been used for medical purposes for many centuries in countries such as India and China for treatment of ulcers, parasitic infections, various skin diseases, psoriasis, arthritis and rheumatism, sinusitis, jaundice and other liver ailments. It has been recognized that most of the therapeutic applications of turmeric is due to curcuminoids, the colouring matter of turmeric, comprising a mixture of curcumin(I), demethoxy curcumin(II) and bis(demethoxy)curcumin(III), the major component being curcumin (Figure 4)[21].

Chemically they can be defined as 1,7- diarylheptanoids having a substituted unsaturated β-diketo moiety in a seven carbon chain with two aryl groups at both ends. There are several reports on the separation of the three curcuminoids from the crude extract using different chromatographic
techniques. They can easily and effectively be separated by column chromatography using different mobile phases including chloroform, acetone, diethyl ether, benzene, etc. Curcumin I is the major component and eluted first from the column followed by curcumin II and curcumin III. Recently, an improved separation of the three curcuminoids was realized using a combination of normal phase column chromatography and phosphate-impregnated preparative-thin layer chromatography\textsuperscript{[22]}. A combination of normal phase column chromatography and phosphate-impregnated preparative-thin layer chromatography technique has been reported as an efficient tool to separate the three curcuminoids\textsuperscript{[23]}.

![Curcuminoids Structure](image)

**Figure 4.** Structure of curcuminoids

Subsequent to the first seminal paper published in “Nature” in 1949, numerous preclinical studies have provided a solid basis for examining efficiency of curcumin against human diseases\textsuperscript{[24]}. More than 15,000 articles published within the past two decades have discussed the molecular basis for the antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, and anticancer activities assigned to curcumin. Clinical trials conducted on this molecule, have shed light on the role of curcumin in various chronic conditions, including autoimmune, cardiovascular, neurological, and
psychological diseases, as well as diabetes and cancer. Common to all of these clinical studies have been the safety, tolerability, and non-toxicity even at high doses. The underlying mechanism for clinical efficiency of curcumin seems to be modulation of numerous signaling molecules. However, because of the complex nature of the diseases, the underlying mechanism in many cases remains unclear\cite{25}.

Literature is extensive on the antitumor activity of curcumin and its structural analogues. In a large number of studies, it was found that these compounds are toxic to cancerous cells and cytoprotective to healthy cells. Various animal models or human studies proved that curcumin is extremely safe even at very high doses. For example, different clinical trials indicated that curcumin, when taken as high as 12 g per day, is well tolerated\cite{26,27}. Similarly, the efficiency of curcumin in various diseases including cancer has been well established. The pharmacological safety and efficacy of curcumin makes it a potential compound for treatment and prevention of a wide variety of human diseases\cite{28}.

Numerous published articles, audio recordings, and videos on this subject are available to the public. Some links to videos about the use and health benefits of turmeric and curcumin are summarised in Table 1.
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Most of the drugs used in chemotherapy are potential poisons at higher doses than prescribed by a physician and most of them have one or other severe side effects. Public awareness about these aspects of chemotherapy increasingly necessitating the search for drugs having natural origin and physiologically active biomolecules are thoroughly investigated by modern techniques to explore their possible applications in the diagnosis and treatment of various diseases. In this context, curcumin has a unique record that more than 15,000 publications have appeared from 1990s to date claiming different beneficial biological effects. The pace of curcumin research is growing rapidly in diverse fields [29].

While the majority of researchers have been pursuing the biological aspects, a few others were interested in understanding the important chemistry of curcumin behind its unique biological activity. Curcumin research has become one of the most favourite subjects in different branches of chemistry. In organic chemistry the extraction and synthesis of curcumin and new synthetic derivatives was the main focus of research. Inorganic chemists have used its metal chelating abilities through the β-diketo group to form new structural entities with modified biochemical activities. Physical chemists have focused on the highly sensitive spectroscopic properties of curcumin to study its interactions with micro heterogeneous systems and biomolecules. Analytical chemists have been employing curcumin’s unique absorption spectroscopic properties to identify and quantitatively estimate trace elements like for e.g., estimation of boron, as a red coloured product. Other chemistry studies that are useful in understanding the biological activity of curcumin are its chemical reactivity with reactive oxygen species (ROS), addition reactions, degradation and formation of nanoconjugates and formulations [30-32].
3. Importance of present investigation

The pharmacological safety and efficacy of curcumin makes it a potential compound for treatment and prevention of a wide variety of human diseases. Unfortunately, its clinical application is restricted by its poor solubility in water, light sensitivity, low absorption and bioavailability. The reasons for reduced bioavailability of any agent within the body are low intrinsic activity, poor absorption, high rate of metabolism, inactivity of metabolic products and/or rapid elimination and clearance from the body [33]. Studies over the past three decades related to absorption, distribution, metabolism and excretion of curcumin have revealed poor absorption and rapid metabolism of curcumin that severely curtails its bioavailability. Problems of curcumin bioavailability such as low serum levels, limited tissue distribution, apparent rapid metabolism and short half-life are to be modified to enhance its bioavailability and potential as a drug [34].

Adjuvants, which can block metabolic pathways of curcumin, are one of the major means that are being used to improve its bioavailability. Nanoparticles, liposomes, micelles, use of absorption factors (e.g. piperidine/piperine), the encapsulation of curcumin in the cavities of cyclodextrins, and phospholipid complexes are other promising novel formulations, which appear to provide longer circulation, better permeability, and resistance to metabolic processes [35].

The chemical structure of curcumin plays a pivotal role in its biological activity. It has three important functionalities: an aromatic o-methoxy phenolic group, α, β-unsaturated β-diketo moiety and a seven carbon linker. Research in the last two decades has provided evidence for the role of these different functional groups in its crucial biological activities. The o-methoxy phenol group and methylenic hydrogen are responsible for the antioxidant activity of curcumin, and curcumin donates an electron/hydrogen atom to reactive
oxygen species. Curcumin interacts with a number of biomolecules through non-covalent and covalent binding. The hydrogen bonding and hydrophobicity of curcumin, arising from the aromatic and tautomeric structures along with the flexibility of the linker group are responsible for the non-covalent interactions\textsuperscript{[36].}

The β-diketo group forms chelates with transition metals, thereby reducing the metal induced toxicity and some of the metal complexes exhibit improved antitumor activity. In order to enhance the biological properties and antitumor activity of curcumin, a large number of curcumin derivatives and analogues have been designed and synthesized through structural modifications such as variation of the aromatic rings and their substituents or replacing the heptadione bridge chain of curcumin and other linkers. Numerous studies dealing with the enhanced biological activity of curcumin derivatives and/or analogues can be found in the literature\textsuperscript{[37, 38].}

Another strategy to improve the biological activity of curcumin was to chelate it with metals. The presence of enolisable β-diketo group in a curcumin molecule makes it an excellent ligand for metal chelation. Several metal chelates of curcumin are reported to possess biological activity over that of free curcumin\textsuperscript{[39].} The past 10 years have witnessed a dramatic increase in studies directed to the synthesis, characterization and biological investigation of metal curcumin complexes. Highly promising results with metal curcumin complexes have also been reported in the fields of antiarthritic/antirheumatic activity, antimicrobial/antifungal activity, anti-viral/anti-HIV activity and biological imaging/radio imaging\textsuperscript{[40].} Copper complexes of curcumin and its derivatives were found to be better antitumor agents than were the parent compounds\textsuperscript{[41].}

Clinical trials are ongoing to test the efficacy of curcumin against a large number of diseases. Intense research is also being undertaken to modify
the structure of curcumin so as to increase the bioavailability and potency while maintaining the relative non-toxic nature of this natural product. Synthesis of compounds structurally related to curcumin by new methods or by reported methods with varying structural features not encountered in natural sources is an active area of research and such studies developed a large number of products with greater beneficial properties than curcumin. Recently there is a surge of activity on preparation and characterization of curcumin-metal complexes due to the strong affinity of β-diketo moiety as an efficient metal chelator. Although it is confirmed that curcumin reduces metal toxicity in living systems through complexation, the actual role of these metal complexes in curcumin biology appears to be complex and unclear. Detailed research is warranted on structure-activity evaluation of the curcumin-metal-complexes in solution[42,43].

4. Description of research problem

We are interested in the development of novel curcumin analogues with improved biological profiles. In our studies, the seven carbon linker in the structure of curcumin molecule is modified by incorporating a cyclopentane ring to the enolizable β-diketone moiety. The reported synthesis of curcumin involves a condensation reaction with appropriate aromatic aldehyde and acetylacetone in its boron chelate form to avoid side reactions at the active methylene group[44]. Most of these methods are time consuming and tedious. In the present investigation, we used conventional reaction under microwave to synthesise these compounds.

Numerous microwave-assisted aldol condensations have been reported but the use of microwave energy in carrying out boron-assisted regioselective aldol condensation was less found in literature. Owing to the simplicity, rapidity, turnover and environment friendliness, use of microwave in organic synthesis has become very popular. Since the reported procedure for synthesis
of analogues of curcumin involved heating conditions, it appeared logical to attempt their synthesis under microwave irradiation conditions. Moderate to excellent yields of the desired compounds were obtained when the reaction mixture was irradiated with microwaves for 3 minutes. It should be noted that the reactions were carried out in a conventional microwave oven and these reactions were qualitatively reproducible.

In the present investigation, the basic curcumin ligand system is modified and a series of unsymmetrical curcumin analogues were synthesized. The modification of the curcumin skeleton included both incorporation of a cyclopentane ring to the enolizable β-diketone moiety which is common to all ligands and a deviation in the nature and position of phenyl substituent, as well as replacing phenyl ring with hetero aryl and naphthyl / substituted naphthyl rings. Copper (II), nickel(II) and zinc(II) complexes of all these ligands were also synthesised. The ligands and their metal complexes were characterized by various physico-chemical methods including electronic, IR, $^1$H NMR, $^{13}$C NMR and mass spectral studies. The ligands were found to be existing in their enolic form and the metal complexes have 1:2 metal ligand stoichiometry.

In view of reported biological activities of curcumin, the possible antioxidant, antitumor and DNA binding properties of both free ligands and their complexes were carried out by standard methods. The results obtained were compared with curcumin reported in the literature. The antioxidant activity of ligands and their metal complexes were studied by measuring DPPH radical scavenging, super oxide radical scavenging, lipid peroxidation inhibitory and hydroxyl radical scavenging activities.

The DNA binding properties with calf thymus (CT) DNA were studied by recording the absorption spectra of the compounds in the absence and presence of CT-DNA, fluorescent quenching studies with ET (Ethidium bromide)-bound CT DNA and by viscosity methods. In vitro cytotoxicity studies were carried out using Dalton’s lymphoma ascites (DLA) cell lines.
For evaluating *in vivo* cytotoxicity, DLA was maintained as ascites tumours in female Swiss albino mice. The effect of compounds on DLA cell induced solid tumour model was also carried out.
REVIEW OF LITERATURE

1. Turmeric

Turmeric (*Curcuma longa L*) is a dietary spice, frequently used in Asian cooking. Turmeric is of special importance to humans with the discovery that its rhizome powder, when added to various food preparations, preserves their freshness and imparts a characteristic flavour and colour \[45\]. It is used as a household remedy for various skin infections and dietary problems since ancient times. In *Ayurveda*, turmeric has been used internally as a stomachic, tonic and blood purifier and externally in the prevention and treatment of skin diseases. Traditional Indian medicinal systems claim the use of its rhizome against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis \[46\]. Modern interest in turmeric began in 1970’s when researchers found evidence suggesting that the herb may possess anti-inflammatory properties and anticancer properties. Products isolated from turmeric showed a strong antioxidant action when tested on the model systems like oxidation of linoleic acid and in vitro peroxidation of brain lipids. A large number of reports and several reviews have appeared on the chemistry, processing and technology of turmeric \[47-49\].

2. Composition of turmeric oil

The aroma of turmeric is due to its volatile oil, while the phenolic compounds and its analogues account for its bright yellow colour. Due to its lower commercial importance, the chemistry of turmeric oil has not received much attention earlier. Kelkar and Sanjeev Rao reported that steam distilled volatile oil is predominantly a mixture of sesquiterpene ketones and alcohols \[50\]. Malingre reported p-cymene, β-sesquiphellandrene, turmerone, ar-turmerone and sesquiterpene alcohols from *Curcuma longa* \[45\]. Chen, Yu and Fangy compared composition of the volatile oils of rhizome and tuber of *Curcuma longa* of Chinese origin. Turmerone (24%), ar-turmerone (8.4%) and
curdione (11.58%) are found to be the major compounds in both the oils. However, ar-curcumene was found in rhizome oil to the extent of 12.2%, but it was not reported in tuber oil\cite{45}.

Kiso, Suzuki, Oshima, and Hikino isolated a new oxygenated sesquiterpene, curlone, from aqueous ethanolic extract of *Curcuma longa* rhizomes\cite{51}. There was considerable quantitative variation in the main components depending upon the cultivars from which the oil was produced. Cooray, Jansz, Ranatunga, and Wimalasena examined the effect of maturity on the major components of the rhizome oil produced from a single turmeric cultivar grown in Sri Lanka, and it was reported that ar-turmerone (24.7–48.9%) and turmerone (20–39%) are the major compounds\cite{52}. Imai, Morikiyo, Furihata, Hayakawa, and Seto reported the two new sesquiterpene keto-alcohols viz. turmeronol A and turmeronol B from the dried rhizomes of *C. longa*\cite{53}. The phytochemicals isolated from turmeric oil are given in Figure 1.
3. Isolation of curcumin

The colouring principle of turmeric was first isolated in 1815 by Vogel and Pelletier and was named as curcumin\(^{54}\). Curcuminoids refer to a group of phenolic compounds present in turmeric, which are chemically related to its principal ingredient curcumin. Three curcuminoids were isolated from turmeric viz., curcumin, demethoxy curcumin and bisdemethoxy curcumin (Figure 4, Section I). All three impart the hallmark yellow pigmentation to its rhizomes. Although the chemical structure of curcumin was determined in 1910, the potential uses of curcuminoids in medicine have been studied extensively from 1970s onwards. These studies increased interest in the isolation of curcumin and several new methods and techniques were applied to isolate curcumin from the rhizomes of turmeric\(^{55}\).
Because curcumin is nearly insoluble in water, most of these methods make use an organic solvent for its isolation. Sastry reported the isolation of curcumin and related demethoxy compounds from turmeric by extraction with organic solvents. The drawback was low recovery of the curcuminoids (1.5–2.0%) \[^{56}\]. Krishnamurthy, Mathew and Nambudiri reported the hot and cold percolation extraction methods with good yields with a high recovery of curcumin\[^{57}\]. Stransky reported that curcumin was isolated from the rhizome by the action of soap solution at 60–90\(^\circ\)C. However, curcumin obtained by this method was found to be a paste, and keeping the solution at alkaline pH at higher temperature may bring structural changes\[^{45}\]. Tonnesen, Karlsen and Aghikary reported the isolation of curcumin by its insoluble lead salt\[^{45}\].

Recently, Baumann, Rodrigues, and Viana have claimed efficient extraction of curcuminoids using supercritical CO\(_2\)\[^{58}\]. Although supercritical fluid extraction is known to be a clean technology giving acceptable yields and purity, its major disadvantage lies in its high operating pressures. The scale up problems could also be severe when the extraction is to be done at large scales.

Dandekar and Gaikar reported microwave assisted extraction (MAE) technique for selective and rapid extraction of curcuminoids\[^{59}\]. Turmeric powder was irradiated for 2 and 4 min with microwave showed marginally higher extraction of curcuminoids. Sodium cumenesulfonate was reported to be an efficient hydrotrope for the extraction of curcuminoids.

In 2000, Anderson, Mitchell, and Mohan reported a technique for isolating curcumin from ground turmeric\[^{22,60}\]. They magnetically stirred the ground turmeric in dichloromethane and heated at reflux for 1 h. The mixture was suction-filtered, and the filtrate was concentrated in a hot-water bath maintained at 50 °C. The reddish–yellow oily residue was triturated with hexane, and the resulting solid was collected by suction filtration. Further TLC analysis (3%methanol–97% dichloromethane) showed the presence of all three
components. Preparative TLC with the crude curcuminoids gives pure curcumin.

Isolation of pure curcumin from plant material is time consuming and pure curcumin sold on the market is therefore, a purified extract containing a mixture of the three curcuminoids i.e. curcumin (75–81%), demethoxy curcumin (15–19%) and bisdemethoxy curcumin (2–6%). Now numerous companies like Sigma-Aldrich Chemicals are marketing curcumin commercially with purity up to 94%.

4. Structural characteristics of curcumin

The chemical structure of curcumin was established in 1910 by Miłobedzka, von Kostanecki and Lampe\textsuperscript{[55]}. On boiling with alkali, curcumin gave vanillic acid and ferulic acids whose structures were established. Fusion with alkali yielded protocatechuic acid and oxidation with potassium permanganate yielded vanillin. On hydrogenation, mixtures of hexahydro- and tetrahydroderivative were obtained. Based on degradative studies, these authors clearly established the identity of curcumin as diferuloylmethane or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione\textsuperscript{[61]}. Like acetylacetone, curcumin displays typical keto–enol tautomerism (Figure 2). However, unlike acetylacetone, the central beta-diketone functionality is flanked by the sterically demanding unsaturated phenolic groups, which results in an unusually wide and flat beta-diketonate ligand. Thus the overall shape of curcumin ligands with the two large wings attached to the beta-diketone unit may be compared to that of an eagle\textsuperscript{[62]}.
Figure 2. Tautomers of curcumin

Tonnesen, Karlsen and Mostad reported the three dimensional molecular structure of curcumin from X-ray crystallographic studies\textsuperscript{[63]}. The single crystal xrd data suggest that in solid state curcumin entirely exist in the intramolecularly hydrogen bonded enol form with trans orientation of the alkenyl groups. In solution it exists as cis-trans isomers where the trans-form in which the two phenolic-methoxy groups are on the opposite sides of the curcumin backbone is slightly more stabilized than the cis-form\textsuperscript{[64]}.

5. Synthesis of curcumin

A century after its isolation from turmeric, the first paper on synthesis of curcumin was reported by Lampe in 1918. The method involved five steps starting from carbomethoxy feruloyl chloride and ethyl acetoacetate\textsuperscript{[65]}. Later, Pabon reported a simple method for the synthesis of curcumin in high yields using acetyl acetone and substituted aromatic aldehydes in the presence of boron trioxide (B\textsubscript{2}O\textsubscript{3}), trialkyl borate and \textit{n}-butylamine\textsuperscript{[44]} and with slight
modifications this method by Pabon has been adopted by several research groups for practically all subsequent curcumin syntheses. Along with the biscondensation product (major product), monocondensation product is also formed in these methods (Scheme 1). The desired biscondensation product can be separated by column chromatography [44].

There are some patents indicating utilization of B₂O₃, trialkylborate and n-butylamine along with inert organic solvents to improve the yields. Attempts to replace boric oxide with boric acid did not prove to be successful. Rao and Sudheer proposed the use of trifluoroboronite and produced stable curcuminoid trifluorboronites that can be hydrolysed in aqueous methanol at pH 5.8 to get curcumin [66]. In all these methods the primary step is the reaction of acetylacetone with suitably substituted aromatic aldehydes. To prevent participation of the diketone in Knoevenagel condensations, it is complexed with boron. Anhydrous conditions and polar aprotic solvents, where curcumin can be separated easily from the reaction mixtures, are suitable for these reactions. Primary and secondary amines are used as catalysts to provide the necessary basicity to deprotonate the alkyl groups of the diketone. To remove the water produced during the condensation reaction scavengers like alkyl borates are employed. Unless removed, the water can react with the diketone complex, thereby reducing the curcumin yield. The boron complex dissociates into curcumin under slightly acidic conditions. Curcumin from this reaction mixture can be separated by repeated precipitation followed by column chromatography [44].
6. Reactivity of curcumin

Important chemical reactions associated with the biological activity of curcumin are the hydrogen donation reactions leading to oxidation of curcumin, nucleophilic addition (Michael reaction) reactions, hydrolysis, degradation and enzymatic reactions. All these have significant role in different biological activities of curcumin.

Reactions with ROS

Curcumin has been found to be an excellent scavenger of most reactive oxygen species (ROS), a property that bestows curcumin with antioxidant activity in normal cells. ROS consists of both free radical oxidants and molecular oxidants. Free radical oxidants participate in hydrogen abstraction and also in electron transfer reactions. All three active sites of curcumin can undergo oxidation by electron transfer and hydrogen abstraction. Detailed
investigations by different groups have confirmed that during free radical reactions, the most easily abstractable hydrogen from curcumin is from the phenol-OH group, resulting in formation of phenoxy radicals, which are resonance stabilized across the keto-enol structure\[^{67-69}\]. The reaction of peroxy radicals with curcumin produces curcumin phenoxy radicals, which are less reactive than the peroxy radicals and thereby cause protection from ROS-induced oxidative stress. The regeneration reaction of phenoxy radicals back to curcumin by water soluble antioxidants like ascorbic acid, impart the molecule with a chain breaking antioxidant ability like vitamin E. Scavenging reactions of several other free radical ROS such as hydroxyl radicals, superoxide radicals and alkoxy radicals by curcumin has been reported\[^{70,71}\].

Among the molecular oxidants, reactions with peroxynitrite, hydrogen peroxide are the most common ones. In several biological models curcumin has been found to protect cells under conditions where there is excessive production of these molecular oxidants\[^{72}\]. However, there are not many studies elucidating the possible chemical reactions and identification of the reaction products. There are few reports in the literature on direct reaction of curcumin with peroxynitrite. The rate constants and the inhibition concentrations of curcumin to prevent nitrotyrosine formation indicate that curcumin is as powerful antioxidant against peroxynitrite-induced oxidative stress\[^{73}\].

Curcumin was found to be an effective antioxidant in different in vitro assays including: reducing power, DPPH\(^*\) (2,2-diphenyl-1-picrylhydrazyl), ABTS\(^{++}\) (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and O\(_2\)\(^{--}\) radical scavenging, hydrogen peroxide scavenging and metal chelating activities\[^{74}\]. Curcumin also showed strong antioxidant activity in different in vivo assays like AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) induced hemolysis in erythrocytes and inhibition of erythrocyte lipid peroxidation\[^{75}\]. Studies on the ability of curcumin to inhibit lipid peroxidation in a variety of models such as rat brain homogenates, rat liver microsomes,
erythrocytes, liposomes, and macrophages provoked a number of recent studies of the antioxidant mechanisms by which the curcuminoids exert their activity, in an attempt to correlate activity with structural features of the molecule. The primary basis for the activity is believed to be the ability of the curcuminoids to scavenge free radicals in vivo, especially peroxo radicals of the form ROO’, where R is an alkyl group. Reactive radicals scavenging and antioxidant activity of curcumin was interpreted as originating by H-atom abstraction from the free hydroxyl group. The phenolic group is essential for the free radical scavenging activity and that the presence of the methoxy group further increased the activity. The transient absorption at 490–500 nm, which they took to be the evidence of phenoxy radicals, and believed that these radicals were formed by a single-electron transfer (SET) mechanism in aqueous-organic solutions.

In order to explain the radical scavenging property of curcumin analogues lacking both phenolic and methoxy groups, another mechanism is proposed involving H-atom transfer (HAT) from a methylene C–H bond (i.e., between the diketo group). This was in part deduced by attributing the transient peak at 490 nm to be that arising from a carbon-centered radical. Loss of a methylenic hydrogen will also cause a fully conjugated chain resulting in stabilization of the carbon-centred radical. The reactivity of curcumin and hence its efficiency as an antioxidant depends on the nature of the free radical which it encounters, as well as the mechanism (HAT or SET) by which it deactivates the free radical.

Chemical degradation and metabolism

Curcumin undergoes chemical degradation in aqueous-organic solutions and the degradation increases as the pH is increased, which is of a serious concern in its applications. Most phenols in solution form polymers over time, but the degradation of curcumin is not through the phenolic group but is rather found to be through the α,β-unsaturated β-diketo moiety. In dilute solutions (i.e., in micromolar solutions) 90% curcumin degrades in 30
minutes. However the percentage degradation will decrease at high concentrations. Several products like feruloylmethane, ferulic aldehyde, ferulic acid, and vanillin were identified (Scheme 2) from a suspension of curcumin in aqueous basic medium at pH=9\textsuperscript{[81]}.

Scheme 2. Chemical degradation of curcumin
Although not fully understood, it is believed that the degradation is by hydrolysis through the diketo moiety. However the degradation is significantly decreased when curcumin is attached to lipids, liposomes, albumins, cyclodextrin, surfactants, polymers and many other macromolecular and microheterogenous systems[82]. Thus has been found to be of great use that stable curcumin solutions could be prepared in culture medium containing 10% Fetal Bovine Serum (FBS) and also in human blood[83].

Curcumin undergoes much faster degradation when exposed to sunlight. It is one common observation that curcumin/turmeric stains can be quickly removed on exposure to sunlight. The colourless products identified during photo degradation of curcumin are vanillin, ferullic acid, and other small phenols, indicating a similar product distribution during photochemical degradation as in chemical degradation in solution[83,84]. This photo degradation involves formation of the excited states of curcumin. The photo degradation is accelerated in presence of TiO₂ nanoparticles, and this method can be employed to remove turmeric stains from cotton fabrics[85].

The metabolism of curcumin in rats and humans produces different products. Two major pathways have been identified in curcumin metabolism, like O-conjugation and reduction. The O-conjugation products are curcumin glucuronide and curcumin sulfate. The reduction products are tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin. Other minor products are dihydrocurcumin glucuronide, tetrahydrocurcumin glucuronide, ferulic acid and dihydroferulic acid [86,87]. The formation of these products has been confirmed by HPLC and mass spectrometry. Although it has been reported that these processes occur enzymatically, the exact enzymes involved are not yet established. Sulphonation of curcumin through human phenol sulfur transferase enzymes and the formation of reduction products through alcohol dehydrogenase is the one that is widely accepted.
Comparing these metabolic products (Scheme 3) with the degradation products (Scheme 2), it appears that simple hydrolytic degradation is prevented in biofluids. Since the degradation may occur through the β-diketo structure, one can presume that in these systems curcumin is not in free form but rather in conjugated form bound to some proteins or other biomolecules, and as the diketo moiety is involved in binding to the proteins, it is not available for hydrolytic degradation [88]. It may also be implied that the specific enzymatic reactions are probably much faster and do not allow the slow hydrolytic degradation, therefore the latter process cannot compete with the former reaction. This leaves a bigger challenge for chemists to understand the differences between degradation and metabolic reactions in terms of kinetic parameters and also identify the crucial mechanism in these reactions [28].

Nucleophillic addition reactions of curcumin

The α,β-unsaturated β-diketo moiety of curcumin participates in nucleophilic addition reactions. This reaction, known as the Michael addition,
occurs between the unsaturated ketone as an acceptor and anions of –OH, –SH, –SeH as donors [28, 88-90]. It is a 1,4-addition reaction and the resultant product formations are mostly irreversible, but they can be made reversible under oxidizing and basic conditions. Since the anions only act as nucleophiles, pH conditions are very important for this reaction to take place. At physiological pH both –OH and –SH are protonated but –SeH can easily undergo deprotonation, therefore it acts as a better nucleophile. This reaction has been reported to be extremely useful to explain the biological chemistry of curcumin in living cells [91, 92].

Reaction of curcumin with biological thiols like glutathione having –SH groups, is of special interest in explaining its bioactivities. A similar reaction has been observed during the inhibition of thioredoxin reductase by curcumin. Thioredoxin reductase is a crucial enzyme involved in maintaining cellular redox homeostasis. The active centre in this enzyme is selenocysteine. The selenol of selenocysteine, being a stronger nucleophile at physiological pH, easily undergoes 1,4-addition with curcumin, forming covalently bonded species. This reaction is speculated to be mainly responsible for the effective inhibition of the thioredoxin reductase enzyme by curcumin. The methylenic hydrogen of the diketo/enol moiety of curcumin can also act as a nucleophile and participate in Michael addition reactions with stronger electrophiles, but such reactions may not have significance in biological systems [93].

Chemistry of curcumin-metal ion interactions

Curcumin forms coloured complexes with boron in presence of dicarboxylic acids like oxalic acid and these reactions are employed for the spectrophotometric estimation of boron as well as oxalic acid [94]. In addition to boron, curcumin forms stable complexes with a range of metals and metalloids. The α,β-unsaturated β-diketo moiety of curcumin displays typical keto–enol tautomerism (Figure 2) and hence curcumin can function as an excellent chelating agent. In the last two decades, many papers and three excellent reviews have been published on metal-curcumin complexes [47-49].
Metal complexation property of curcumin can lower metal induced toxicity. Metal complexes of curcumin have greater significance in view of the pathology of Alzheimer’s disease, where it has been found that due to its lipophilic nature, curcumin can cross the blood brain barrier and chelate metal ions that are toxic to the neurons. It has also been observed that the incidence of Alzheimer’s disease is significantly reduced among people that are known to regularly consume turmeric in their diet. Curcumin forms stable complexes with all the metals involved in Alzheimer’s disease\cite{95-98}.

Metal coordination of curcumin occurs through the enolic group, where the enolic proton is replaced by the metal ion and the \( o \)-methoxy phenolic moiety remains intact in the complexes. The metal-oxygen bond is characterized by IR absorption at \( \sim 455 \text{ cm}^{-1} \) corresponding to \( \nu(C-O) \) and the carbonyl peaks in the complexes show a small shift of \( \sim 10-20 \text{ cm}^{-1} \) on coordination to metals. Changes in NMR chemical shifts of curcumin have also been reported on metal coordination. The shifts however depend on the affinity and thermodynamic stability of the resulting complexes. In the case of strong complexes, the resonances of protons attached to the double bonds of the alkyl chain show significant downfield shifts, while the penolic protons show negligible shifts in the \( ^1\text{H-NMR} \) spectra, and the \( ^{13}\text{C-NMR} \) spectrum shows down- and up-field shifts of carbons near the coordination site\cite{69}.

Typical structure of curcumin complex with a divalent metal ion is given in Figure 3
A variety of metal ions can be complexed by the β-diketo-moiety of curcumin. The metal complexes so formed often possess a higher stability than the easily degraded curcumin itself. The first complexes of curcumin with medically valuable transition metals, such as Pd, Pt, Rh, and In, were published in 1997[99]. In most cases the resulting complexes exhibited favorable biological activity compared with the parent ligands for a number of molecular targets[100].

There are several papers published in the literature on complexes of curcumin with transition metals like Fe$^{3+}$, Mn$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Cd$^{2+}$, Ru$^{3+}$, Re$^{3+}$ and many others. Complexes with non-transition metal ions and rare earth ions like Al$^{3+}$, Ga$^{3+}$, Sm$^{3+}$, Eu$^{3+}$, Dy$^{3+}$, Y$^{3+}$, Se$^{2+}$ and metal oxides like VO$^{2+}$ have also been synthesized. The structure and physical properties of these complexes depend on the nature of the metal ion, as well as the metal-ligand stoichiometry, which in turn decides their stability and reactivity[101].

Stable 2:1 complexes of some transition metals can be prepared by mixing stoichiometric amounts of curcumin and metal salts in suitable organic solvents and refluxing for few hours, the complex can be separated as a precipitate, and purified by repeated crystallization[102,103].
7. Biological activities of curcumin

The biological characteristics of curcumin were scientifically identified in the mid-twentieth century. In a paper published in Nature in 1949, Schraufstatter and co-workers reported that curcumin is a biologically active compound that has antibacterial properties\[24\]. These authors found that curcumin was active against strains of *Staphylococcus aureus*, *Salmonella paratyphi*, *Trichophyton gypseum*, and *Mycobacterium tuberculosis*. However, research on curcumin became quiet for the next two decades. Curcumin again became the subject of scientific investigation in the 1970s. During this decade, three independent groups discovered diverse characteristics of curcumin, including its cholesterol-lowering, antidiabetic, antiinflammatory, and antioxidant activities\[104\].

Ghatak and Basu showed that curcumin is more potent than hydrocortisone for inhibiting formalin-induced arthritis in rats and carrageenin-induced rat paw edema\[105\]. Later, Srimal and Dhawan reported that curcumin is as potent as cortisone or phenylbutazone for the inhibition of carrageenin-induced rat paw edema. These studies indicate curcumin's potential as an anti-inflammatory agent\[106\]. Singh and Aggarwal demonstrated that curcumin exhibits anti-inflammatory activity by suppressing the proinflammatory transcription factor nuclear factor (NF)-κB and outlined the molecular mechanism of the inhibition\[107\]. The anticancer activity of curcumin was demonstrated in the 1980s by Kuttan and colleagues in both *in vitro* and *in vivo* models\[108\]. Interest in curcumin research has increased substantially over the years.

As on January 2016, more than 6500 articles on curcumin were listed in the National Institutes of Health PubMed database (www.ncbi.nlm.nih.gov/sites/entrez). The pleiotropic activity of curcumin in animal models of many human diseases is now reported by numerous groups. In human clinical trials, curcumin has been found to be safe and efficacious,
and the U.S. Food and Drug Administration has approved curcumin as a “generally regarded as safe” compound.

**Antimicrobial activity**

The antimicrobial activity of α,β-unsaturated carbonyl compounds has been recognized generally due to their ability to react with sulfhydryl containing system essential for normal metabolism of microbes. Several studies have reported the broad-spectrum antimicrobial activity for curcumin including antibacterial, antiviral, and antifungal activities. Because of the extended antimicrobial activity of curcumin and safety property even at high doses assessed by clinical trials in human, mixture of curcumin with other antimicrobial agents is used for the development of antimicrobial skin gels and emulsions with improved skin protection and wound dressing properties.[109]

Composition of curcumin with hydrogel silver nanoparticles is used to increase the function of hydrogel silver nanocomposites for antimicrobial applications and wound dressing. Curcumin-loaded myristic acid micro emulsion with the 0.86 μg/mL of curcumin suitable for skin consumption inhibited 50% of the growth of *S. epidermidis*, one of the nosocomial infectious agents. It showed 12-fold stronger inhibitory effect compared to curcumin activity dissolved in DMSO.[110]

Outburst of drug resistant microbial strains necessitates the studies for synergistic effects of antibiotics in combination with plant’s derivatives to develop the antimicrobial cocktail with a wider spectrum of activity and reduction of adverse side effects of antimicrobial agents. The synergistic activity of curcuminoids and ampicillin combination demonstrated pronounced reduction in the MIC of ampicillin against clinical strain.[111] The consumption of turmeric during the treatment of *S. aureus* infections with these antibiotics especially cefixime can be possibly helpful. Curcumin also demonstrated a synergistic effect in combination with some antibiotics, including ampicillin, oxacillin, and norfloxacin against methicillin-resistant *S. Aureus* strain (MRSA). The synergistic effect of curcumin with ciprofloxacin
against MRSA has also been reported, although there is an evidence of its antagonistic activity against *S. typhimurium* in combination with ciprofloxacin [112,113].

Complexes of curcumin with cobalt nanoparticles showed increased antibacterial activity against *E. coli*. Additionally, fabrication of silver nanocomposite films impregnated with curcumin showed the stronger antibacterial activity against *E. coli* [114]. It was shown that the bactericidal activity of sodium carboxymethyl cellulose silver nanocomposite films (SCMC SNCFs) as an effective antibacterial material was improved by loading of curcumin with SCMC SNCFs [115]. The novel curcumin encapsulated chitosan-[poly (vinyl alcohol)] silver nanocomposite films with pronounced antimicrobial exhibition against *E. coli* proved to be potential antibacterial material for treating infections or wound dressing [116].

Lack of effective therapeutics for the most of viral diseases, emergence of antiviral drug resistance, and high cost of some antiviral therapies necessitate finding new effective antiviral compounds. It has been demonstrated that curcumin has a wide range of antiviral activity against different viruses. Inosine monophosphate dehydrogenase (IMPDH) enzyme is suggested as a therapeutic target for antiviral and anticancer compounds [117] due to its rate-limiting activity in the *de novo* synthesis of guanine nucleotides. In *in vitro* studies, curcumin showed strong inhibitory activity against IMPDH suggesting that it can be a potent antiviral compound via this process. Integrase, an essential enzyme for HIV-1 replication was found to be inhibited by curcumin. The study of energy minimization and the structural analogues of curcumin elicited that an intramolecular stacking of two phenyl rings of curcumin is possibly responsible for anti-integrase activity via bringing the hydroxyl groups into close proximity. The clinical trial of ethanol extract of *C. longa* rhizome on HIV patients reduced the wound infections and considerable decrease in itching symptom and it also affected the abscess to convert to dryness scabs within two weeks [118]. Curcumin showed the anti-influenza
activity against influenza viruses PR8, H1N1, and H6N1. The results showed more than 90% reduction in virus yield in cell culture in presence of curcumin[119].

Substances and extracts isolated from plants have always been a rich arsenal for controlling the fungal infections and spoilage. Due to extensive traditional use of turmeric in food products, various researches have been done in order to study the turmeric and curcumin with the aspect of controlling fungal related spoilage and fungal pathogens. The study of addition the turmeric powder in plant tissue culture showed that turmeric at the 0.8 and 1.0 g/L had appreciable inhibitory activity against fungal contaminations[120]. In vitro screening of the antifungal activity with curcumin against Aspergillus niger, Aspergillus flavus, Aspergillus heteromorphus and Penicillium verruculosum was reported.

Antioxidant activity of curcuminoids

The oxygen consumption inherent in cell growth leads to the generation of a series of ROS. They are continuously produced by the body’s normal use of oxygen such as respiration and some cell-mediated immune functions. ROS include free radicals such as superoxide anion radicals (O$_2^-$), hydroxyl radicals (OH’) and non-free radical species such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen. ROS play a positive role in energy production, phagocytosis and regulation of cell growth, intercellular signalling, and synthesis of biologically important compounds. However, ROS may also be very damaging; they can attack the lipids of cell membranes and DNA. The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases. ROS are continuously produced during normal physiologic events and are removed by antioxidant defence mechanisms. It is well known that ROS are closely involved in various human diseases such as Alzheimer's disease, cancer, inflammation, rheumatoid arthritis and atherosclerosis. Excessive production of the free radical NO in the
brain has been shown to induce neurotoxicity. Typical for neurodegenerative diseases is progressive loss of the structure or function of neurons and eventually even the death of neurons\textsuperscript{[121]}.

Thus, antioxidants are considered to be important as neuroprotective agents. It is commonly recognized that antioxidants can neutralize potentially harmful reactive free radicals in body cells before they cause lipid and protein oxidation and may reduce potential mutation and therefore, help prevent cancer or heart diseases. Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. Currently, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are widely used in the food industry. However, restriction on the synthetic antioxidants is being imposed because of their toxicity to liver and carcinogenicity. Therefore, the development and utilization of more effective antioxidants of natural origins are desired\textsuperscript{[122,123]}. Curcumin was found to be an effective antioxidant in different \textit{in vitro} assays and \textit{in vivo} assays.

\textbf{Cytotoxic activities of curcuminoids}

Cancer is the leading cause of death in developed countries and the second-largest cause of death in developing countries after cardiovascular diseases. More than 200 types of cancers are known in humans, depending on tissue and cell type. Carcinogenesis is a complex process but may be broadly considered to be comprised of three main phases: initiation, promotion, and progression. These closely related steps: going from a normal cell to a transformed initiated cell (initiation); from initiated to pre-neoplastic cell (promotion); and from preneoplastic to neoplastic (progression). There is suggestive evidence that inflammation may have a role in the three phases of carcinogenesis\textsuperscript{[124]}. Cancer initiation has been produced by oxidative stress and chronic inflammation. Inflammation acts a key regulator in promotion of these initiated cells, possibly by providing them with proliferating signals and by preventing apoptosis. Inflammatory response produces cytokines which act as
growth and/or angiogenic factors leading transformed cells to proliferate and undergo promotion. The most clinically established cancer treatment strategies are surgery, radiotherapy, and chemotherapy [125].

The chemotherapeutic approach uses a combination of drugs with various mechanisms of action to enhance the therapeutic efficiency. Currently available monotargeted cancer therapeutics has numerous adverse effects and is expensive. Curcumin is a nontoxic natural product known to exert a wide range of pharmacological activities through its effect on several targets including transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and cellular signaling molecules. These multi target interactions of curcumin form the molecular basis of anti-carcinogenic and chemopreventive effects of curcumin [126].

The anticancer properties include suppression of cellular transformation, prevention of cancer cell proliferation, and suppression of carcinogenic effects. Recent studies have found that curcumin has a dose dependent chemopreventive effect in several animal tumour bioassay systems including colon, duodenal, stomach, esophageal and oral carcinogenesis. It has been shown to reduce tumours induced by benz(a) pyrene and 7, 12-dimethyl benz(a) anthracene, tumor promotion induced by phorbol esters on mouse skin, on carcinogen-induced tumorigenesis in the fore stomach and N-ethyl-N'-nitro-N-nitrosoguanidine-induced duodenal tumours. Curcumin administration during both the initiation and post initiation periods significantly inhibited colon tumorigenesis [127,128]. In addition, administration of the synthetic curcumin in the diet during the promotion/progression stage significantly suppressed the incidence and multiplicity of noninvasive adenocarcinomas and also strongly inhibited the multiplicity of invasive adenocarcinomas of the colon. Curcumin has been demonstrated to induce apoptosis in a variety of cells including prostate cancer cells [129].
Curcumin has been reported to possess anticarcinogenic properties in several experimental models. Because of its lack of toxicity, its efficacy in inhibiting tumorigenesis in several models, and its multiple mechanisms of action, curcumin has been selected for further evaluation by different investigators all over the world. In these studies curcumin and its synthetic analogues were screened for numerous cancer types found in humans. In vitro and animal studies have revealed that curcumin suppresses carcinogenesis and inhibits the proliferation of a wide variety of tumor cells and modulates growth of tumour cells through regulation of multiple cell signaling pathways including cell proliferation pathway. It blocks transformation, tumour initiation, tumour promotion, invasion, angiogenesis, and metastasis. It has shown to display chemotherapeutic and chemopreventive effects in diverse cancers. It inhibits the cell cycle progression of colon cancer cells.

Probably the first indication of curcumin’s anticancer activities in human participants was shown in 1987 by Kuttan and co-workers, who conducted a clinical trial involving 62 patients with external cancerous lesions. Topical curcumin was found to produce remarkable symptomatic relief as evidenced by reductions in smell, itching, lesion size, and pain. Although the effect continued for several months in many patients, only one patient had an adverse reaction. Since then, curcumin, either alone or in combination with other agents, has demonstrated potential against colorectal cancer, pancreatic cancer, breast cancer, prostate cancer, multiple myeloma, lung cancer, oral cancer, and head and neck squamous cell carcinoma.

Curcumin has also found to be a potential for the prevention and treatment of colorectal cancer (CRC) in combination with other agents. Curcumin was administered to patients with CRC after diagnosis and before surgery. Curcumin (360 mg in a capsule form) was given three times a day for 10–30 days. Curcumin administration increased body weight, increased the
number of apoptotic cells, and enhanced the expression in tumor tissue. The curcumin treatment can improve the general health of CRC patients.\textsuperscript{[133]}

Curcumin induces cell death in numerous animal and human cell lines, including leukemia, melanoma, and carcinomas of the breast, lung, colon, kidney, ovaries and liver.\textsuperscript{[134]} Certain data have demonstrated that curcumin exhibits a biphasic action: Low doses lead to oxidative stress and apoptosis, while higher doses lead to reduced production of reactive oxygen species, reduction of ATP and necrotic cell death.\textsuperscript{[135]} It also appears to be able to cause cell death in various cell lines resistant to apoptosis, possibly by activating cell death mechanisms. Apoptosis is a process of programmed cell death through cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay that occurs in multicellular organisms. Necrosis is a form of cell injury which results in the premature death of cells in living tissue by autolysis. It is caused by factors external to the cell or tissue, such as infection, toxins, or trauma which result in the unregulated digestion of cell components.

**Anti-Alzheimer’s disease activity**

Alzheimer’s disease is the most common cause of dementia among people of age 65 or older. It appears well established that the gradual loss of brain function characteristic for Alzheimer’s disease is connected to two main forms of nerve damage. It has been demonstrated that the incidents of Alzheimer’s disease among elderly people of age 70–79 in rural India, who eat curry dishes on a daily basis, is about 4.4 times lower than that of Americans of the same age. Curcumin shows an array of activities that can be helpful in ameliorating Alzheimer disease symptoms acting on various target sites. The therapeutic benefits of curcumin for Alzheimer diseases appear multifactorial via regulation of transcription factors, cytokines and enzymes. It has also been shown to suppress oxidative damage, inflammation, cognitive deficits, and amyloid accumulation in Alzheimer diseases. Furthermore, various in vivo
studies have provided supporting evidence for the therapeutic potential of curcumin in Alzheimer diseases. These studies indicate that curcumin probably interferes with the formation of plaques and can therefore improve the disease condition \[136,137\].

The currently available treatments for this disease have numerous adverse effects, thus underscoring the need for alternative approaches. A randomized, double blind, placebo-controlled study was conducted in the United States to evaluate the safety and tolerability of curcumin in patients with mild to moderate Alzheimer’s disease. This study included the safety, tolerability, pharmacokinetics, and efficiency of curcumin in patients with Alzheimer’s disease, as well as the effects of curcumin on biomarkers associated with the pathology of this disease. It was found that curcumin administration was associated with an increase in vitamin E level, and curcumin did not cause any adverse effects\[138\]. The anti-oxidant activity of curcuminoids might decrease the need for anti-oxidant vitamin E. These observations support the opening of a clinical trial of curcumin against Alzheimer’s disease using large numbers of patients \[139\].

**Anti-inflammatory effects**

Curcumin inhibits cyclooxygenase2 (COX-2) as well as lipoxygenase (LOX), two enzymes involved in inflammation. Indeed, cytokine-induced COX-2 transforms arachidonic acid in prostaglandins during acute inflammatory episodes. COX2 is also prevalent during chronic inflammations\[140\]. Lipoxygenase transforms arachidonic acid in leukotrienes, which take part in leukocytes recruiting and play a role in inflammation. Moreover, curcumin protects keratinocytes and fibroblasts against H₂O₂-induced damages and allows reduction of oxidative and inflammatory stress. Pancreatitis improves after curcumin treatment, which blocks key inflammatory signals. However, by verifying the effect of curcumin in galactose-induced cataract and discovered that small doses of curcumin (0.01%) could increase oxidative stress in hyperglycaemic rats.
Antidiabetic properties

Curcumin has been shown to be effective against diabetes in patients and in experimental animal models. In rats with alloxan-induced diabetes, in streptozotocin (STZ)-induced rats models, and in STZ-nicotinamide–induced rats models, oral administration of various dosages of curcumin was able to prevent loss of body weight; reduce levels of glucose, hemoglobin, and glycosylated hemoglobin in the blood; and improve insulin sensitivity. In rat models of high-fat diet induced insulin resistance and oral administration of curcumin showed an antihyperglycemic effect and improved insulin sensitivity, which was attributed by its anti-inflammatory properties [141,142]. Diabetes mellitus is a chronic metabolic disease in which a person has high concentrations of blood sugar. Because of its anti-inflammatory property, curcumin represents a promising therapeutic option for Diabetes mellitus. Curcumin’s ability to decrease blood sugar levels in human patients was first reported in 1972. Injection of turmeric or curcumin along with insulin synergistically reduced the blood sugar level [143].

Antiarthritic/antirheumatic activity

Arthritis is a chronic disease that results from the inflammation of one or more joints. It usually results from dysregulation of pro-inflammatory cytokines and pro inflammatory enzymes that mediate the production of prostaglandins and leukotrienes, together with the expression of adhesion molecules and matrix metalloproteinases. Although more than 100 different kinds of arthritis have been reported, the three most common forms are osteoarthritis, rheumatoid arthritis, and gout.

The potential of curcumin against arthritis was first reported in 1980 curcumin’s efficiency was compared with that of the prescription drug phenylbutazone [144]. Patients were randomly assigned to receive either curcumin (1.2 g/day) or phenylbutazone (0.3 g/day) for 2 weeks. Curcumin was well tolerated, had no adverse effects, and exerted an antirheumatic activity identical to that of phenylbutazone as shown by improvement in joint
swelling, morning stiffness, and walking time. Curcumin alone and in combination with diclofenac sodium was found to be safe and effective with rheumatoid arthritis [145].

The water insolubility and low bioavailability of curcumin in cells have prompted researchers to develop new formulations based on biocompatible organic substances like liposomes, polyethylene glycols, biopolymers, cellulose, corn oil, hydrogels etc. All these systems have not only shown improved water solubility but also increased curcumin bioavailability. Interestingly the fluorescence of curcumin gets enhanced once solubilised in any of these systems, making it easy to estimate its binding efficiency [146].

Due to their biocompatibility all these systems could be successfully investigated for anti-cancer activity in cancer cells, and in vivo systems, where significant increase in the anticancer activity due to improved bioavailability of curcumin was reported. Liposomal curcumin was found to be the best for improving the bioavailability of curcumin in cells and products based on liposomal formulations are being marketed for different dietary applications of curcumin. Till recently the word nanocurcumin referred to curcumin-loaded organic formulations only. Recently, a large number of inorganic nano formulations with application in delivery of curcumin [146].

Mesoporous silica nanoparticles (MSN) are one of the most employed nanosystems for improving the bioavailability of poorly water soluble drugs. Such systems can be easily manipulated for improved delivery, activity and specificity [147,148]. Due to their ordered nanoporous structures, high surface areas, large pore volumes and high surface densities of hydroxyl groups, MSNs can be functionalized easily. They are biocompatible and they are commonly used in many biomedical applications. Curcumin binds covalently through a silicon-oxygen bond at the diketo moiety. Curcumin-loaded MSNs have been prepared and employed in several studies. In these systems, curcumin release could be controlled for even up to several hours along with improvement in the stability and bioavailability of curcumin [149].
The fluorescence of curcumin is enhanced on MSN conjugation and therefore has the potential to be employed for imaging biomolecules/organelles. Novel cyclodextrin functionalized MSN have been found to be photothermally controlled on exposure to light to release curcumin on demand in zebra fish larve. Large amount of curcumin could be loaded into spherical microcapsules containing L-lysine, trisodiumcitrate and silica sol (colloidal suspension). These microcapsules could be triggered to release curcumin by adjusting the pH to acidic conditions. MSN-curcumin conjugates, increased the cytotoxicity of curcumin in HeLa cell lines (derived from cervical cancer cells) and also in normal fibroblast cell lines. They also increased photocytotoxicity of curcumin in human oral cancer cells, on exposure to light\textsuperscript{[150,151]}. 

Gold nanoparticle-based curcumin formulations have been prepared and reported recently. Gold nanoparticles find application in biology and medicine, for drug delivery, diagnosis and cancer treatment. In a simple method, curcumin-gold composites were prepared by mixing alkaline curcumin solutions with gold salts, where the ionized curcumin acts both as a reducing agent and also as the capping agent. In this case, both the phenolic-OH and enolic-OH donate hydrogen for reduction of Au\textsuperscript{3+} ions. Such gold-curcumin conjugates were reported to be hemocompatible and non-toxic \textsuperscript{[152]}. 

The spectrum of beneficial biological activities of cucumin reported so far is very wide. Excellent reviews and monographs are available on this subject. Some significant biological activities reported are briefly summarized below (Figure4).
8. Synthetic analogues of curcumin

Curcumin is reported to have a large number of beneficial biological activities. Since biological activities are directly related to the molecular structure, structurally related compounds can also be ideal candidate for therapeutic applications. This aspect along with the flexibility in the structure of curcumin with two aryl groups and a seven carbon linker lead to the synthesis of a large number of synthetic analogues of curcumin with a variation in the aryl substituents / aryl rings. A large number of such compounds are reported in the past two decades (Table 2). Biological screening with some of these compounds and their metal derivatives gave promising results.
Table 2. Synthetic analogues of curcumin

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9. Biological activities of metal complexes of curcumin and its synthetic analogues

Curcumin-metal complexes not only modify the physico-chemical properties of curcumin but they also affect the biological reactivity. In general it has been observed that complexation with curcumin reduces the toxicity of the metals and some curcumin complexes with metals like Cu$^{2+}$, Mn$^{2+}$, act as new metal-based antioxidants. Due to the reversible electron transfer reactions with superoxide ions, Cu$^{2+}$ and Mn$^{2+}$ complexes of curcumin behaves like superoxide dismutase enzyme [161].

One of the first biological investigations on metal curcumin complexes was published as early as 1987 and comprises an antiarthritic study of the orally active gold(III) curcumin complex [Au(Curc)$_2$]Cl (I). This complex was prepared in a simple manner by mixing curcumin with gold(III) chloride in a 2 : 1 molar ratio in ethanol. *In vivo* antiarthritic activity was reported to this five co-ordinated Au$^{3+}$ complex [162].
Several metal chelates of curcumin are reported to possess biological activity over that of free curcumin. Divalent and trivalent metal complexes of synthetic curcumin analogues given in Table 2 are synthesised and characterized by our group during 1995-2010. John and Krishnankutty studied the antitumor activities of curcumin, piperonylcurcumin, 2-hydroxy naphthylcurcumin, cinnamylcurcumin, and their copper complexes\textsuperscript{[154,157]}. Copper complexes of curcumin and its derivatives were found to be better antitumor agents than were the parent compounds. Further, the curcumin-copper complex was equally effective as curcumin against cadmium induced oxidative damage in mice. Theoretical calculation of ionization energies of curcumin and curcumin-copper complex(II) have shown that these possess higher reactive oxygen species scavenging ability than does curcumin\textsuperscript{[163]}. 
Similarly, it was demonstrated that curcumin-manganese complex (III) exhibited a more potent neuroprotective activity than curcumin both in vitro and in vivo suggesting that this complex may be useful as a neuroprotective agent in the treatment of acute brain pathologies associated with NO-induced neurotoxicity and oxidative stress-induced neuronal damage such as epilepsy, stroke, and traumatic brain injury\textsuperscript{[161]}. Studies by Sui, Salto and Li showed that the modest activity of curcumin as an in vitro inhibitor of HIV-1 and HIV-2 proteases is enhanced more than 10-fold when curcumin is complexed with boron (IV and V)\textsuperscript{[164]}.

Recently 2:1 complexes of curcumin with Ga\textsuperscript{3+} (VI) have been prepared with high radiochemical purity. These compounds have also been reported to
be binding to β-amyloid fibrils very strongly, with possible applications in the diagnosis of Alzheimer’s disease\cite{165}.

\begin{center}
\includegraphics[width=0.5\textwidth]{Fig1.png}
\end{center}

VI

It was revealed that curcumin strongly interacts with the Al\textsuperscript{3+} ion. Curcumin is thus capable of scavenging Al\textsuperscript{3+} and preventing this metal ion from interacting with proteins like β-amyloid, thereby weakening the β-amyloid toxicity and oxidative stress. 1:1 Al\textsuperscript{3+}-curcumin complex (VII) showed less affinity to DNA binding than free Al\textsuperscript{3+}, which has been attributed to its ability to reduce development of Al\textsuperscript{3+} induced Alzheimer’s disease\cite{166, 167}.

\begin{center}
\includegraphics[width=0.5\textwidth]{Fig2.png}
\end{center}

VII

A vanadylecurcumin complex [VO(Curc)\textsubscript{2}] (VIII) was reported to show a 2-fold increase in antirheumatic activity and a 4-fold increase in inhibiting smooth muscle cell proliferation as compared to free curcumin \textit{in vitro}. Further, this complex was more effective as an anticancer agent, compared to uncomplexed curcumin\cite{168}.
Zn\(^{2+}\)-curcumin complexes showed anti-cancer, gastro protective and antidepressant effects in rats\(^{[169]}\).

Two copper complexes Cu(Et\(_2\)Curc)\(_2\) (dioxane) (X) and Cu(Bu\(_2\)Curc)\(_2\) (XI) have large two-photon absorption cross-sections, a property desirable for bioimaging of living cells and tissues. Compared with single-photon absorbing materials, the molecular excitation by the simultaneous absorption of two photons presents several advantages that include high confined excitation capacity, intrinsic three-dimension resolution, and the possibility of imaging at an increased penetration depth in tissue, with reduced photo-damage and background fluorescence. The experimental results showed that the complexes exhibit a large two-photon absorption crosssection in the near-infrared region,
high quantum yield and photostability and low cytotoxicity. The in vitro study utilized the human breast cancer MCF-7 cell line that was imaged by two-photon fluorescence microscopy\textsuperscript{[170]}. The tumor targeting capability of (X) and (XI) on tumor-bearing nude mice in vivo demonstrated its high targeting capability to test cancerous cells. The results suggested that these Cu(II) complexes are promising probes for in vivo imaging\textsuperscript{[171]}.
Heteroleptic complexes containing suitable spectator ligands in addition to curcumin like \( \text{VO(Curc)(phenanthroline)Cl} \) (X) have also been reported recently found to possess more activity than \( \text{VO(Curc)_2} \) (XII)\(^{[172]} \).

Most of these mixed ligand complexes of curcumin apparently contain only one curcumin ligand and ligands like pyridine, 2,2'-bipyridine, phenanthroline, terpyridine derivatives, tertiary phosphines, cyclopentadienyl ligands and \( \eta^6 \)-coordinated arenes like cymene or hexamethylbenzene as the co-ligands. A few examples of these mixed ligand complexes are summarized here.

Experimental and theoretical studies on two rare earth metal curcumin complexes, \( \text{La(Curc)_3(pyridine)_2} \) (XIII) and \( \text{Eu(Curc)_3(pyridine)_2} \) (XIV), revealed their potential application as a biological fluorescent probe\(^{[173]} \).
Photodynamic therapy is a modern minimally invasive technique for the treatment of cancer. It has many benefits compared with chemotherapy, radiotherapy, or surgery including reduced long-term morbidity, no resistance development and no negative consequences of repeated treatments. The three basic components of any photodynamic approach are light, oxygen, and the photosensitizer. The most widely used photosensitizer in current clinical use is photofrin, a tetrapyrrole-containing compound. However, this compound has several disadvantages, e.g., long-lasting skin photosensitivity and low absorbance at $\lambda = 630$ nm. Hence there is great interest in alternatives which offer advantages over photofrin, including absorption between $\lambda = 600$ nm and 800 nm, rapid clearance from non-pathological tissue, and low toxicity in the absence of an optical trigger. Huang et al made an interesting modification of the curcumin skeleton by bridging the curcumin moiety via 1,6-dibromohexane and a hydroxyl naphthyl-group to a porphyrin molecule[174,175]. The ligand and its Ni(II), Cu(II) and Zn(II) complexes (XV-XVIII) showed strong binding interactions with DNA and light-triggered cleavage activity[176].
Complexes of curcumin-and 4,4'-bipyridine with Zn$^{2+}$ were more effective than curcumin to kill neuroblastoma cells$^{[177]}$. The presence of intrinsic fluorescence in (bipy-9)Zn(Curc) (XIX) and (bipy-CH$_2$OH)Zn(Curc) (XX) allows the combination of anticancer properties with an excellent tool for investigating their mechanism of action through optical methods in a single molecule, without additional external agents.
The ionic tetrafluoroborate Zn(II) complex [(bipy-9)Zn(Curc)]BF₄ (XXI) and the neutral phenanthroline based Zn(II) derivative (phen)Zn(Curc)Cl (XXII) also showed potential growth inhibition in *in vitro* studies[177].

Fluorescent curcumin-metal complexes are being explored for imaging of cancer cells. Re(CO)₃(Curcumin)(H₂O) (XXIII) complex is fluorescent and show affinity to β-amyloid plaques, which has potential to be explored in microscopic imaging of the tissue of Alzheimer’s disease patients. Similarly ⁹⁹Tc(CO)₃(curcumin)(H₂O) (XXIV) complexes have been produced in high radiochemical yield, and showed significant affinity to β-amyloid plaques and such systems are being developed as novel radio diagnostic agents for Alzheimer’s disease[178, 179].
Two monophosphine and bis(phosphine)curcumin rhenium carbonyl complexes fac-Re(CO)$_3$(PPh$_3$)(Curc) (XXV) and cis–trans-Re(CO)$_2$ (PPh$_3$)$_2$ (Curc) (XXVI) were found to show selective binding to β-amyloid plaques of Alzheimer’s disease and stain the β-amyloid plaques, allowing clear visualization of the plaques$^{[180]}$. 

A DNA docking study of (η$^6$-p-cymene)RuCl(Curc) (XXVII) revealed the same mechanism of action that has been established in Pt chemotherapy. The ruthenium η6-p-cymene-PTA-curcumin complex (PTA = 1,3,5-triaza-7-phosphaadamantane) (XVIII) showed not only superior solubility properties
due to the sophisticated ligand design but also superior cytotoxicities compared to other classes of related compounds \[181\].

Recently a few mixed ligand complexes of curcumin with lanthanides having unique chemical and biological activities have been reported. Curcumin-terpyridyl-lanthanide complexes (XXIX and XXX) showed enhanced photocytotoxicity in HeLa cells. Mixed ligand-curcumin complexes of ruthenium metal showed antibacterial activity. It has significant potential for photo chemotherapeutic applications and highest toxicity for the colorectal tumour cell lines \[182,183\].
Numerous reports on both homoleptic and heteroleptic metal complexes of synthetic analogues of curcumin are coming up, each claiming with improved biological activities or other properties that have potential applications in the diagnosis and treatment of various diseases.
MATERIALS, METHODS AND INSTRUMENTS

Materials

All chemicals used for synthesis were of analytical reagent grade procured from Merck, India and used as such. Solvents used were of commercial purity grade procured from commercial sources such as Nice/ Loba Chemicals and were purified by standard procedures\textsuperscript{[184]}. For recording UV Visible spectra and for chromatographic analysis, spectroscopic grade solvents procured from Merck, India was used.

2-acetylcyclopentanone used for synthesis of ligands was procured from Sigma Aldrich, USA. Copper (II) acetate monohydrate, nickel (II) acetate tetrahydrate, and zinc (II) acetate dihydrate were used for the synthesis of metal complexes. Double distilled water collected from all glass equipment was used in all preparations.

For DNA binding studies, ethidiumbromide (EB), tris(hydroxymethyl) aminomethane(Tris) and calf thymus (CT) DNA were procured from Sigma Aldrich, USA. Tris–HCl buffer solution was prepared using de-ionized and triple distilled water using a quartz water distillation setup.

For antioxidant assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitrobluetetrazolium (NBT) were procured from Sigma Aldrich, USA. Sodium dodecyl sulphate, thiobarbituric acid, ascorbic acid, deoxyribose, and riboflavin were procured Himedia Chemicals.

For screening antitumor activity, cyclophosphamide (cycloxan, Biochem. Pharmaceutical Industrial Ltd.) is used as control. Carboxymethyl cellulose and the dye trypan blue were procured from Himedia Chemicals.

Dalton’s lymphoma ascites (DLA) cells were obtained from the Cancer Research Institute, Mumbai, India. DLA was maintained as ascites tumors in
Swiss albino mice, purchased from Kerala Agricultural University, Thrissur, Kerala. They were fed with normal mouse chow (Lipton India) and water *ad libitum*.

**Instruments**

Instruments used in this investigation are

1. Elemental Vario EL CHN analyzer
2. Heraeus Elemental analyzer
3. AAS (Perkin Elmer 2380)
4. Jasco V – 550 UV visible spectrophotometer
5. Jasco FTIR-4100 Fourier transform infrared spectrophotometer
6. BrukerAvance III, 400MHz FT NMR spectrometer
7. JEOL / SX-102 MS
8. ELICO-CM-82 T conductivity bridge
9. Varian E-12 ESR spectrometer
10. PCI-analytic sonicator
11. LG-Highwave (70W) microwave oven
12. MPF-4 fluorescence spectrophotometer
13. Ostwald’s viscometer
14. ELICO SL 218 double beam UV-VISIBLE spectrophotometer
15. Rohan India Ltd. Hemocytometer
16. Gouy’s type magnetic balance
Methods

Microwave assisted synthesis were carried out in a domestic microwave oven (70W) as the source of microwave. PCI-analytic sonicator (6.5L, 200W) is used for sonication.

Molecular mass of compounds reported were determined by Rast’s method using camphor as medium.

Carbon and hydrogen percentages reported are by microanalysis carried out at SAIF IIT Bombay, Mumbai and at CDRI Lucknow, India.

The electronic spectra of the ligands and complexes were recorded from solutions (10^{-3}M) using CHCl₃ or DMSO as solvents.

The IR spectra were recorded in the region 4000–250 cm⁻¹ in KBr discs from STIC, CUSAT. Bands were calibrated using the nearest polystyrene bands.

The $^1$H NMR and $^{13}$C NMR spectra were recorded in CDCl₃ or DMSO d₆ from SAIF-IIT Bombay and SAIF - IIT Madras, Chennai. Chemical shifts are given in ppm relative to tetramethylsilane.

FAB mass spectra were recorded at room temperature using argon (6 KV,10 mA) as the FAB gas and 3-nitrobenzyl alcohol as the matrix. ESI-MS were recorded (scan range 30-2000) in positive ion mode with accelerating voltage 10 KV. ESR spectra of copper complexes were recorded at 77 K.

Molar conductivity measurements were recorded in DMF at 28± 1°C using solution of about $10^{-3}$ M in a cell having cell constant 0.51cm⁻¹.

Magnetic susceptibility measurement on powder samples were carried out at room temperature (28 ± 1°C) using mercury tetrathiocyanatocobaltate (II) as standard.
The lipid peroxidation inhibitory activity was determined by the thiobarbituric acid method by recording absorbance at 532 nm.

The DPPH free radical scavenging activity was monitored by measuring the absorbance of 0.5 mM DPPH solution at 520 nm in the presence of test compounds at different concentrations ranging from 10-100 µg/ml after incubating for 30min at 37°C in the dark. The EC$_{50}$% (Efficient Concentration of the test compound necessary to decrease the initial DPPH radical concentration by 50%) value was determined.

The super oxide radical scavenging activity was determined by the NBT reduction method.

The hydroxyl radical scavenging was measured by studying the competition between deoxy ribose and the test compounds for hydroxyl radicals generated from Fe$^{3+}$/ ascorbate/ EDTA/ H$_2$O$_2$ system using TBA.

UV–vis absorption spectrophotometry was used to monitor the interactions of ligand and its complexes with CT-DNA at 7.2 pH in double distilled water containing tris-(hydroxymethyl)amino methane (Tris, 10$^{-2}$ M). The binding experiments were carried out by recording the absorbance changes on adding increasing concentrations of DNA (0 – 250 µM) against a fixed concentration of the ligand and its complexes (50 µM) at room temperature (28 ± 1°C).

Viscosity measurements of 1x10$^{-4}$ M CT-DNA in Tris–HCl/NaCl buffer were performed using an Ostwald viscometer at 35 ± 0.2 °C in a thermostatic water bath. Flow time was measured with a digital stopwatch. Each experiment was performed three times and an average flow time was calculated.
Since the copper(II) complexes are non-luminescent at room temperature, in fluorescence quenching studies, EB-bound CT-DNA solution in Tris–HCl/NaCl buffer is used.

\textit{In vitro} cytotoxicity studies were carried out using DLA cells. The stained and unstained cells were counted using heamocytometer using trypan blue as the staining agent.

For \textit{in vivo} studies, groups of Swiss Albino mice (female, 6 per group) were injected intraperitonially (ip) with Dalton’s lymphoma ascites tumour cells \((1\times10^6 \text{ cells/animal})\). They were injected ip with test compound suspended in carboxy methyl cellulose (CMC) and the injections of the test compounds were started 24 hours after tumor inoculation and continued for 10 consecutive days.

Solid tumors were also induced in groups of Swiss albino mice (female, 6 per group) by subcutaneous injection of DLA cells \((1\times10^6 \text{ cells/animal})\) on the right hand limb. One group was kept as control and other groups were injected intramuscularly (im) with the test compounds \((25\text{mg/kg body weight})\) and the injections of the test compounds were started 24 hours after inoculation and continued for 10 consecutive days. Tumor diameter was measured every third day for one month and the tumor volume was calculated.

The detailed procedures of all biological studies were presented in \textbf{Results and Discussion, Part B} of the thesis.